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Elucidating mechanisms of toxic action of dissolved organic chemicals in oil sands process-affected water (OSPW)

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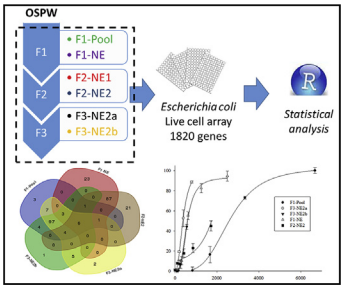
HIGHLIGHTS

- Six genes were uniquely responsive to acutely toxic extracts of OSPW.
- Gene enrichment analysis demonstrated a role for oxidative stress, protein and DNA damage.
- Roles of sulphur- and nitrogen-containing chemicals in acute toxicities of extracts of OSPW.

GRAPHICAL ABSTRACT



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ABSTRACT

Oil sands process-affected water (OSPW) is generated during extraction of bitumen in the surface-mining oil sands industry in Alberta, Canada, and is acutely and chronically toxic to aquatic organisms. It is known that dissolved organic compounds in OSPW are responsible for most toxic effects, but knowledge of the specific mechanism(s) of toxicity, is limited. Using bioassay-based effects-directed analysis, the dissolved organic fraction of OSPW has previously been fractionated, ultimately producing refined samples of dissolved organic chemicals in OSPW, each with distinct chemical profiles. Using the *Escherichia coli* K-12 strain MG1655 gene reporter live cell array, the present study investigated relationships between toxic potencies of each fraction, expression of genes and characterization of chemicals in each of five acutely toxic and one non-toxic extract of OSPW derived by use of effects-directed analysis. Effects on expressions of genes related to response to oxidative stress, protein stress and DNA damage were indicative of exposure to acutely toxic extracts of OSPW. Additionally, six genes were uniquely responsive to acutely toxic extracts of OSPW. Evidence presented supports a role for sulphur- and nitrogen-containing chemical classes in the toxicity of extracts of OSPW.

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1. Introduction

Oil sands process-affected water (OSPW) is a complex mixture produced as a waste stream of the open-pit surface mining oil sands industry where hot water is used to extract bitumen from the oil sands. Consisting of high concentrations of dissolved bitumen-derived organic chemicals, dissolved salts and metals, and suspended particulate matter, OSPW exhibits both acute and chronic toxicity to a range of organisms (Anderson et al., 2011; Hao et al., 2005; Morandi et al., 2015). Because of concerns about adverse effects in the environment, the oil sands industry follows a no-release policy and OSPW is stored on-site in large tailings ponds where it is recycled back into the extraction process. Eventually, all process-affected materials must be remediated and returned to the surrounding environment. However, acceptable remediation techniques and criteria for evaluating toxic potency prior to release of treated OSPW remain to be established.

Currently it is known that the dissolved organic fraction of OSPW is responsible for most toxicity, however the specific compounds and mechanisms by which these chemicals cause toxicity remains an active field of research (Anderson et al., 2011). Recently, by use of a bioassay effects-directed analysis approach, and ultra-high resolution mass spectrometry (uHRMS) for characterization of OSPW organic fractions, it was demonstrated that acute toxicity of OSPW was due primarily to naphthenic acids (NA), oxy-NAs, but also other neutral and basic chemical species containing sulphur and nitrogen (Morandi et al., 2015, 2016). It has been suggested that the mechanism of acute toxicity is due to non-polar narcosis (Frank et al., 2008; Morandi et al., 2016, 2015; Scarlett et al., 2012). Genotoxicity has been observed in Rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to a synthetic OSPW extract (Lacaze et al., 2014). Additionally, the test systems SOS Chromotest and Ames test have identified concentrated whole extracts of OSPW as genotoxic and mutagenic (Zetouni et al., 2017). However, evidence exists to support the role of other mechanisms of OSPW toxicity (Wiseman et al., 2013a,b).

High throughput, open format investigations into mechanisms of action of chemicals have gained popularity over recent years as new technologies have emerged (He et al., 2012b; Jung et al., 2017; Zhang et al., 2011). Previous application of RNAseq to investigate mechanisms of toxicity of OSPW collected from West In-pit tailings pond (WIP) following 7-days of exposure of male fathead minnow (*Pimephales promelas*; FHM) demonstrated response of genes related to oxidative metabolism, oxidative stress, apoptosis and immune function (Wiseman et al., 2013b). These results are consistent with those observed previously during exposures of embryos of FHM to OSPW which resulted in differential expression of several genes related to oxidative stress and apoptosis, and resulted in greater peroxidation of lipids in exposed embryos (He et al., 2012a). Additional evidence exists to support oxidative stress playing a role in toxicity of WIP-OSPW following exposure of the midge *Chironomus dilutus* (*C. dilutus*) since changes to abundances of transcripts of genes involved in responses to oxidative stress, and peroxidation of lipids were observed in larvae compared to control (Wiseman et al., 2013a).

Naphthenic acids contribute to acute toxicity of OSPW, and NA standards and commercial NA mixtures have been used as surrogates to represent OSPW derived NAs (Hughes et al., 2017; Morandi et al., 2016, 2015; Rowland et al., 2011; Zhang et al., 2011). The live cell genome reporter assay has previously been used to identify a number of novel molecular mechanisms of toxicity of a commercial NA mixture. Differentially expressed genes could be identified in the Pentose Phosphate Pathway, ATP-binding cassette transporter complex and SOS response pathway (Zhang et al., 2011). In this work, the live cell array genome reporter system was used to screen acutely toxic organic extracts of OSPW produced in previous work of Morandi et al. (2015), that were prepared from Basemine Lake OSPW (formerly WIP) collected in 2012, to elucidate molecular mechanisms of toxic action.

2. Materials and methods

2.1. Data compilation

Recently, an effects directed analysis of dissolved organic fraction of OSPW provided better understanding of chemical classes that contribute to acute lethality of exposed aquatic organisms (Morandi et al., 2015). In that study, three rounds of sequential fractionation and toxicity testing were completed, ultimately producing five fractions with observed acute toxicity to embryos of FHM and *Vibrio fisheri*; a pooled sample representative of the whole dissolved organic fraction of OSPW (F1-Pool), round one neutral extractable fraction (F1-NE), round two acidic fraction (F2-NE2) and round three early and late eluting fractions by use of HPLC (F3-NE2a and F3-NE2b) and a second round fraction with no observed LC50 (F2-NE1). Additionally, the chemical profile of each extract was determined by use of uHRMS. Therefore, fractions produced as part of the effects-directed analysis were screened to investigate potential molecular mechanisms of toxicity of the whole mixture, represented by the complete mixture of OSPW (F1-Pool), and how its toxicity is related to toxicity of refined extracts of OSPW. To the knowledge of the authors, this is the first application of open format investigations of molecular mechanisms of toxicity to be conducted in conjunction with effects-directed analysis.

2.2. Sample collection and chemical fractionation

A brief overview of the chemical characterization procedure is presented in the supporting information and fractionation methodology in Morandi et al. (2015). Gravimetric mass of each extract was measured following extraction to establish the concentrations of extracts. Chemical characterization data of each extract was compiled from Morandi et al. (2015) and are presented in Fig. S1.

2.3. Escherichia coli K-12 strain MG1655 gene reporter system

The gene reporter system was purchased from Open Biosystems Thermo Fisher Scientific (Huntsville, AL, USA), which was developed at the Weizmann Institute of Science. Assays were conducted as outlined in Zhang et al. (2011). Briefly, 1855 promoter clones (out of 2500 in the whole genome) were grown at 37 °C in a 1× LB-Lennox media with 25 μg/mL kanamycin for 24-h prior to assay. Therefore, this assay facilitates the measurement of promoter activity by use of fluorescence to monitor gene expression. Individual wells are used for each promoter clone to facilitate the measurement of 1855 individual genes over time. Assays of cytotoxicity were performed prior to performing the genome reporter assay. Cytotoxicity assays were performed in 96-well plates by use of the parent strain, and exposures were performed in triplicate to four concentrations of fractions for 24 h. For completion of the genome reporter assay, concentrations equivalent to the twenty-percent inhibition of growth (IC20) for cytotoxicity, were used for the F1-Pool, F1-NE, F2-NE2, F3-NE2a and F3-NE2b samples. Due to lack of observed toxicity, the F2-NE1 sample was assayed at a concentration of 392.5 mg/L, which was the highest tested concentration. Black 384-well optical bottom plates (NUNC, Rochester, NY, USA), were prepared with 71.25 μL of LB medium per well and incubated for 3.5 h prior to exposure. Following incubation, optical density (OD) of each well was measured at 600 nm by use of a Synergy H4 hybrid microplate reader (BioTek Instruments Inc., Winooski, VT).

Following the initial reading, 3.75 μL of nanopure water (control) or sample of OSPW was added to each well to a final volume of 75 μL. The intensity of GFP of each well was quantified consecutively every 10 min during the 4 h exposure (excitation/emission: 485 nm/528 nm).

2.4. Statistical analysis

Statistical analyses were completed by use of R 2.3.0 (R 2.3.0, Vienna, Austria). IC20 was calculated by use of the probit model. To assess responses of the gene reporter system, linear regressions were used to assess effects of time ($p \leq 0.001$) for the response of each gene. Details on the statistical analyses applied have been previously published (Guan et al., 2016; Jung et al., 2017; Zhang et al., 2011). Effects on expression were expressed as fold-change relative to control. Fold changes of 1.5- and 2-fold change were used as cut-off values for gene selection for downstream analysis described below.

2.5. Network visualization, pathway analysis and clustering analysis

Lists of genes were developed by use of cutoffs of 1.5- or 2.0-fold changes. The transcriptional network was constructed by use of the ClueGO plug-in of Cytoscape v2.3.3, an open source bioinformatics software platform by use of 1.5-fold gene lists. The ClueGO v2.2.3 enrichment/depletion two-sided hypergeometric test was conducted by use of the Bonferroni step-down correction and used ($p < 0.05$) to identify enriched Gene Ontology (GO) terms/Biological Processes and Kyoto Encyclopedia of Genes and Genomes pathways (KEGG). Hierarchical cluster analysis (HCA), and Principal component analysis (PCA) were completed by use of R.2.3.0. For PCA, lists of genes identified by use of biological or KEGG pathway analyses were linearly combined as suggested previously (Ma and Dai, 2011) prior to analysis. For analysis of uHRMS data, chemical classes were limited to those accounting for a minimum of 5% of total ion count in at least one sample, resulting in a list of 27 chemical classes (Fig. S1). To facilitate easier interpretation of score and loadings plots following PCA analysis, identified biological processes and KEGG pathways, and chemical classes were labelled with identifiers (Table S1 and S2).

3. Results and discussion

3.1. Cytotoxicity of OSPW fractions

Fractions of OSPW were cytotoxic within the range of tested concentrations (Fig. S2) and IC20 values are presented (Table 1). In general, *E. coli* was less sensitive to extracts of OSPW than the marine bacterium, *Vibrio fisheri or* embryos of fathead minnow (Morandi et al., 2015). However, *E. coli* exhibited sensitivity previously observed following exposure to a technical mixture of NAs (Sigma Aldrich # 70340) (Zhang et al., 2011). Maximum inhibition of growth of *E. coli* exposed to F1-Pool, F1-NE, F2-NE2, F3-NE2a and F3-NE2b was 100, 72.9, 22.9, 82.5, 88.8 and 52%, respectively. In contrast, no significant toxicity was observed following exposure to F2-NE1. The rank-order of toxicity was similar to that observed by Morandi et al. (2015), however, in this study, fraction F2-NE2 exhibited the greatest toxic potency, whereas Morandi et al. (2015) previously observed that fraction F3-NE2a exhibited the greatest toxic potency. Nevertheless, exposure of *E. coli* to the F1-Pool, F1-NE, F2-NE1, F2-NE2, F3-NE2a and F3- NE2b extracts of OSPW resulted in similar classification of samples as toxic (i.e. observed acute lethality) and non-toxic (i.e. no observed acute lethality) as previously observed (Morandi et al., 2015) and demonstrated the utility of this *E.coli* cell line for screening toxicity of dissolved organic chemicals from OSPW.

3.2. Changes to gene expression following exposure of E.coli to extracts of OSPW

Exposure of *E.coli* to fractions of OSPW resulted in differential expressions of 263 genes when a 1.5-fold cut-off was applied (Table S3). When a 2-fold cut-off was applied, 115 genes were identified as being differentially expressed (Table 2, Fig. 1).

Six differentially expressed genes were unique to the five fractions of OSPW that were acutely toxic (i.e. F1-Pool, F1-NE, F2-NE2, F3-NE2a, F3-NE2b) (Table 3 and Fig. 2). The six genes could be classified into four broad categories based on their biological functions; transcriptional regulators (*yceP, clpB*), enzyme or putative enzymes (*adhE, ykgE*), putative surface protein (ybjE), and outer membrane phospholipase (pldA). The gene *yceP* was down-regulated greater than 2-fold following exposure to all five acutely toxic fractions of OSPW. Down-regulation of expression of *yceP* is associated with the general stress response of *E.coli* resulting in biofilm formation, increased motility, and catabolite repression (Domka et al., 2006). The gene *clpB* encodes for a chaperone protein that has been associated with proteotoxicity following exposure to heat stress (Thomas and Baneyx, 2000). The enzyme, alcohol/acetaldehyde dehydrogenase (*adhE*) is involved in maintenance of cellular redox homeostasis (Echave et al., 2003). Additionally, the enzyme (*ykgE*) has an oxido-reductase function and is involved in maintenance of the cellular redox environment (Rodriguez and Atsumi, 2014). The surface protein (*ybjE*) is an important transmembrane transporter of lysine (Pathania and Sardesai, 2015). Phospholipase A (*pldA*) is important in maintaining homeostasis of cell functions, and down-regulation of the *pldA* gene might indicate reduced requirements for precursors for production of cyclooxygenases and lipoxygenases that ultimately are utilized for production of prostaglandins, and which are important for control of multiple biological processes (Hardaway and Buller, 1979). Only one gene, *lacZ,* which encodes for the protein, β-galactosidase, and is involved in metabolism of lactose was similarly differentially expressed among all six fractions of OSPW (Juers et al., 2012).

Table 1
Concentrations of dissolved organic chemicals in OSPW required to inhibit growth of *E.coli* wild-type strain by twenty percent (IC20).

Sample	IC20 (mg/L) (95% CI)
F1-Pool	1608 (786–2429)
F1-NE	1057 (993–1057)
F2-NE1	N/A
F2-NE2	152 (87.82–217.9)
F3-NE2a	212 (170–254)
F3-NE2b	372 (306–438)

Table 2
Numbers of genes differentially expressed ≥1.5- and 2- fold in E.coli exposed to fractions of OSPW relative to *E. coli* exposed to a solvent control.

Sample	1.5-fold		Total	2-fold		Total
	Up	Down		Up	Down	
F1-Pool	2	121	123	0	27	27
F1-NE	20	98	118	6	23	29
F2-NE1	14	78	92	8	19	27
F2-NE2	32	88	120	16	29	45
F3-NE2a	0	121	121	0	37	37
F3-NE2b	6	111	117	2	22	24

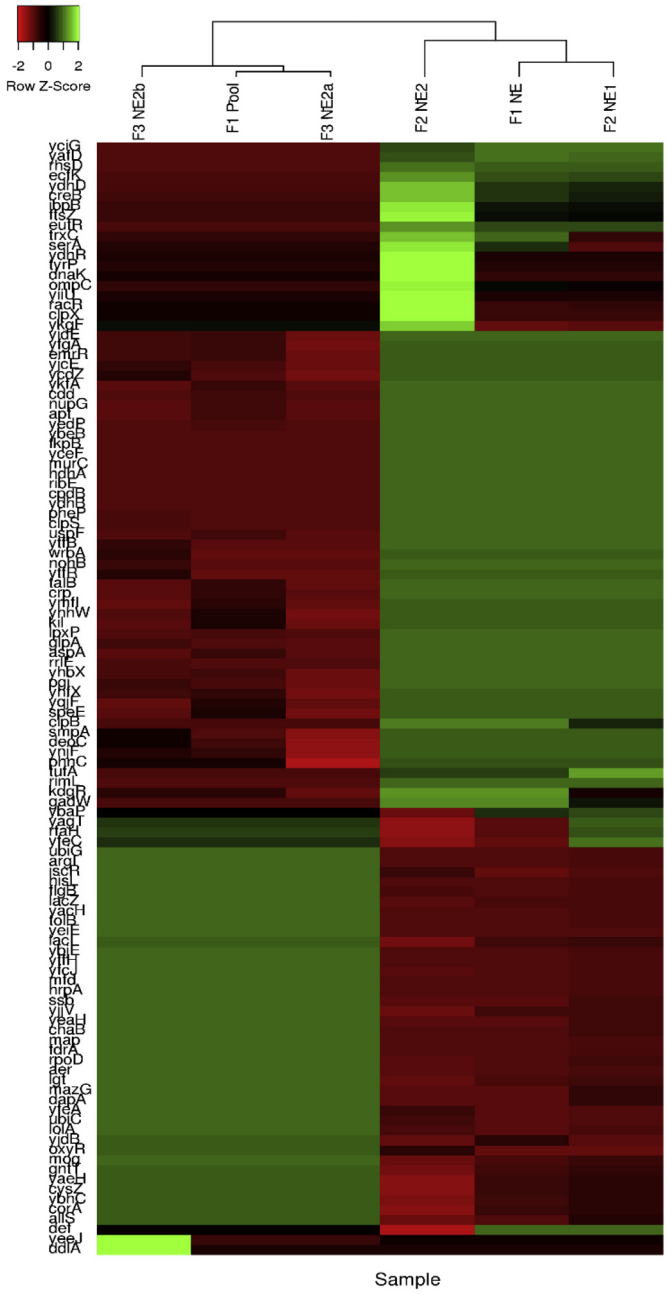

Fig. 1. Hierarchical cluster analysis and heat map for genes differentially expressed ≥ 2-fold in *E. coli* exposed to extracts of OSPW relative to *E. coli* exposed to a solvent control. Z-score represents standardized data, red infers down-regulation and green infers up-regulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Genes differentially expressed in *E. coli* exposed to each of the acutely toxic fractions of OSPW.

Gene name	Description	Biological function
yceP	Transcription repressor	Biofilm formation
clpB	Transcription repressor	Chaperone protein
ykgE	Enzyme	Putative surface protein
pldA	Enzyme	Outer-membrane phospholipase
adhE	Enzyme	Alcohol dehydrogenase
ybjE	Putative surface protein	L-lysine transmembrane transporter

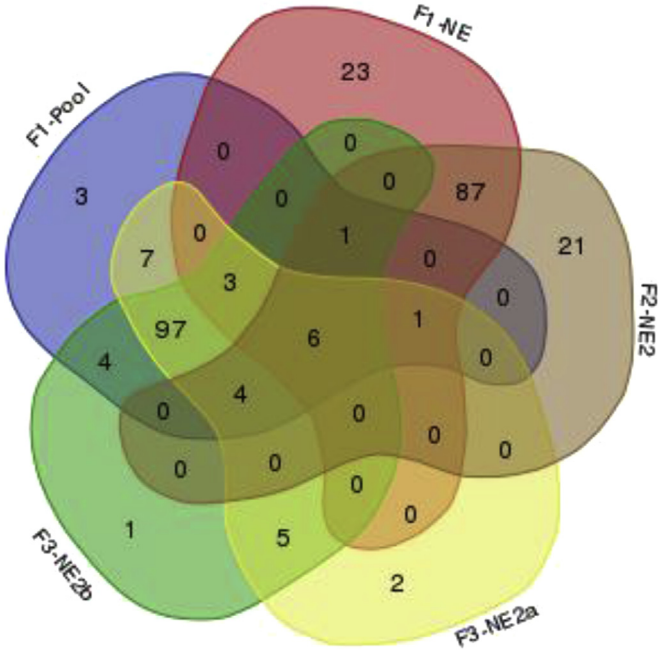

Fig. 2. Venn diagram comparing genes expressed in *E. coli* exposed to acutely toxic fractions of extracts of OSPW.

3.3. Stress responsive genes affected by exposure to fractions of extracts of OSPW

Lists of differentially expressed genes of *E. coli* exposed to each fraction of OSPW were compared to a set of 93 genes previously identified as related to the general stress response of *E. coli* (Gou et al., 2010). Overall, 11 of the stress-response genes were found to be responsive to extracts of OSPW (Table S2). Genes related to energy stress (*bola, crp*) and protein stress (*clpB*) were differentially expressed in *E. coli* exposed to F1-Pool (Table S2). Additional stress pathways included cell death, detoxification, redox stress, drug resistance and DNA repair, and are similar to results of Zhang et al. (2011).

3.4. Mechanisms of toxic action of extracts of OSPW

Gene enrichment analysis identified 20 responsive biological and KEGG pathways in *E. coli* exposed to fractions of OSPW (Table S1, Fig. 3). Among samples, 6, 5, 3, 8, 7 and 7 biological processes or KEGG pathways were identified as responsive to F1-Pool, F1-NE, F2-NE1, F2-NE2, F3-NE2a and F3-NE2b fractions, respectively ($p < 0.05$). Because F1-Pool contains the whole extractable organic fraction of OSPW, it was instructive to identify mechanisms of toxicity for this sample. Negative regulation of cellular processes (GO: 0048523) was identified as a biological process that was responsive in *E. coli* exposed to F1-Pool and likely representative of non-specific toxicity of extracts as suggested previously (Jung et al., 2017). This conclusion was supported by greater than 2-fold down-regulation of the global transcriptional regulator *crp*, which has been identified as responsive to general stress. Additional changes in gene expression were associated with regulation of anabolic and catabolic processes in cells. Of particular interest were changes to regulation of metabolism of organic hydroxyl compounds (GO: 1901615) and the KEGG pathway Pentose Phosphate Pathway (KEGG id: 00030), which might suggest response to changes in redox status of cells. A number of genes associated with responses to oxidative stress and anabolism were

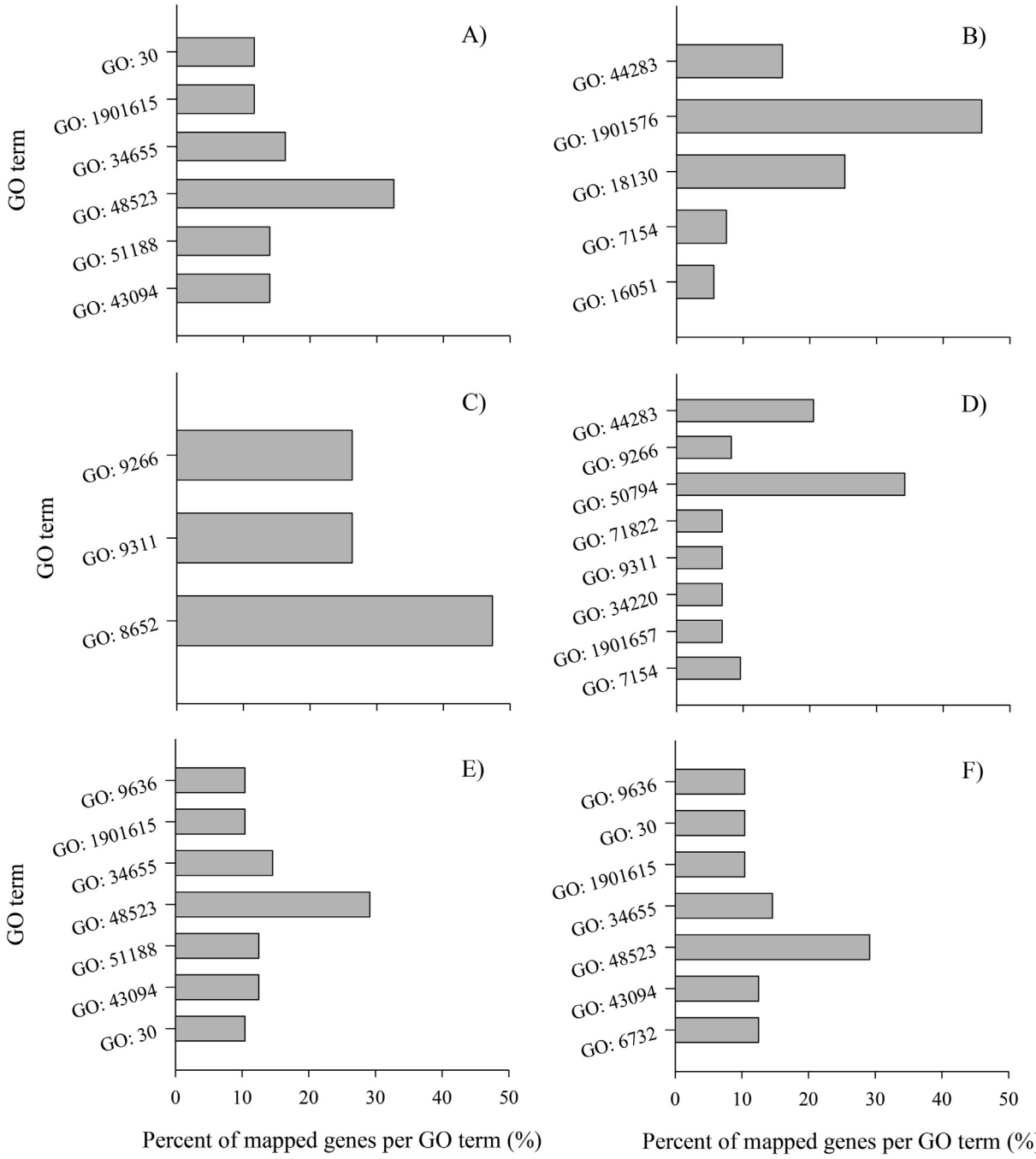

Fig. 3. Proportions of differentially expressed genes mapped to GO biological processes or KEGG pathway for *E. coli* exposed to: A) F1-Pool, B) F1-NE, C) F2-NE1, D) F2-NE2, E) F3-NE2a or F) F3-NE2b. Biological processes identified include: carbohydrate biosynthetic process (GO: 0016051), cell communication (GO: 0007154), cellular amino acid biosynthetic process (GO: 0008652), cellular metabolic compound salvage (GO: 0043094), coenzyme metabolic process (GO: 0006732), cofactor biosynthetic process (GO: 0051188), glycosyl compound metabolic process (GO: 1901657), heterocycle biosynthetic process (GO: 0018130), ion transmembrane transport (GO: 0034220), negative regulation of cellular process (GO: 0048523), nucleobase-containing compound catabolic process (GO: 0034655), oligosaccharide metabolic process (GO: 0009311), organic hydroxy compound metabolic process (GO: 1901615), organic substance biosynthetic process (GO: 1901576), Pentose Phosphate Pathway (KEGG id: 00030), protein complex subunit organization (GO: 0071822), regulation of cellular process (GO: 0050794), response to temperature stimuli (GO: 0009266), response to toxic substance (GO: 0009636), small molecule biosynthetic process (GO:0044283).

identified in the two pathways. Alcohol dehydrogenase, which is encoded by the gene *adhE,* has a putative role under aerobic conditions as a member of the antioxidant defense system and is a putative helicase (Echave et al., 2003). Octaprenyl diphosphate synthase (*ispB*) is essential for growth and catalyzes a reaction in the production of ubiquinone, an important component of the antioxidant defense system (Søballe and Poole, 1999).

Previously, the Pentose Phosphate Pathway of *E. coli* MG1655 was identified as being responsive to a commercial mixture of NAs (Zhang et al., 2011). Exposure of *E. coli* to the F1-Pool resulted in greater than 2-fold down-regulation of *pgi* and *talB*. Phosphoglucose isomerase (*pgi*), catalyzes interconversion of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) as part of the oxidative branch of the Pentose Phosphate Pathway and is

important in control of metabolite flux between glycolytic and Pentose Phosphate Pathways (Krüger et al., 2011). Down-regulation of *pgi* has been associated with greater production of reducing equivalents, such as nicotinamide adenine dinucleotide phosphate (NADP+) in response to oxidative stress (Krüger et al., 2011). *Trans*-aldolase B (*talB*) catalyzes a reversible reaction of the non-oxidative branch of the Pentose Phosphate Pathway, where down-regulation coincides with general negative regulation of cellular processes, as the non-oxidative branch of the Pentose Phosphate Pathway produces important intermediates for normal metabolic function (Frederiks et al., 2008). These results were interesting because results of several studies have suggested that oxidative stress likely is a mechanism of toxicity of OSPW(He et al., 2012a; Wiseman et al., 2013a,b).

3.4.1. Hierarchical cluster and principal component analysis of genomic data

No biological or KEGG pathway was identified as responsive to all fractions of OSPW that exhibited acute toxicity (Fig. S3). PCA was used to investigate relationships between toxicity ranking of fractions (i.e. most toxic fraction has rank equal to one) and identified mechanisms of toxicity (Fig. 4). Cell communication (GO: 0007154) and negative regulation of cellular processes (GO: 0048523) were associated with a lesser toxicity rank (i.e. more toxic extracts) (Fig. 4). HCA and PCA clustered samples similarly, grouping extracts into two groups: 1) F1-Pool, F3-NE2a and F3-NE2b; 2) F1-NE, F2-NE1 and F2-NE2 (Fig. 1, Fig. S4).

Clustering of samples was instructive, since F1-Pool is representative of the whole dissolved organic fraction of OSPW, whereas F3-NE2a and F3-NE2b are the most refined and toxic fractions of OSPW produced by Morandi et al. (2015) accounting for less than 11 and 8% of the organic mass in the F1-Pool, respectively. Because

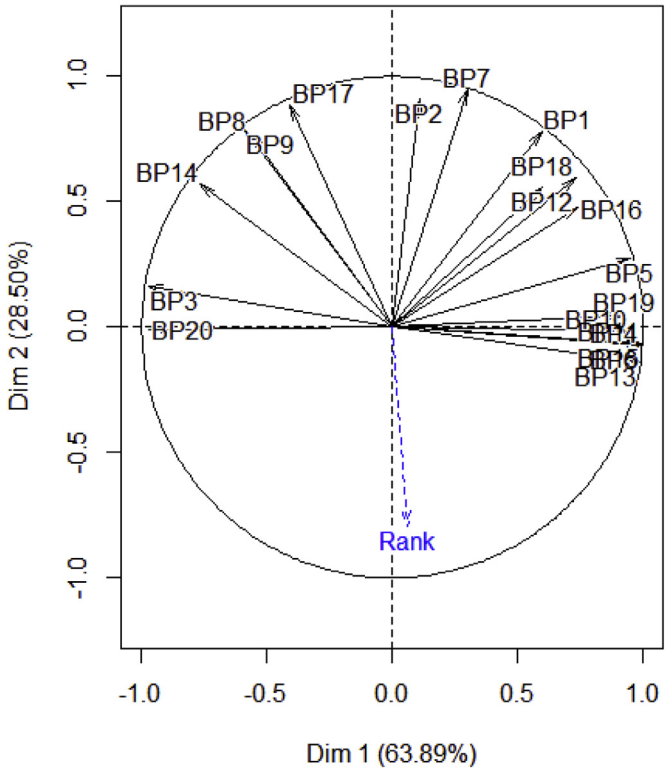

Fig. 4. Variable factor map of differentially expressed biological pathways in *E. coli* following exposure to fractions of extracts of OSPW. Biological process (BP) labels are presented in Table S1.

dose-response curves for acute lethality of embryos of FHMS to F3-NE2a and F3-NE2b were different, it has been suggested that these fractions differ in their mechanisms of acute toxicity (Morandi et al., 2015). However, as demonstrated in Fig. S2, dose-response curves were similar for *E.coli* and the number of common genes (Fig. 2), biological pathways (Fig. 3, Fig. S3) and clustering by use of HCA (Fig. 1) and PCA (Fig. S4) suggest similar mechanisms of toxicity in *E. coli* exposed to these fractions.

3.5. Effect of compositions of fractions on mechanisms of toxicity of extracts of OSPW

Identification of chemical species and chemical classes responsible for toxicity of OSPW has received much attention recently (Morandi et al., 2015; Peng et al., 2016; Scarlett et al., 2013; Yue et al., 2014). Therefore, it was instructive to investigate if profiles of relative proportions of chemicals in extracts could be used to classify samples as toxic or non-toxic, and if clustering was similar to the results of biological pathway analysis. Extracts of OSPW had differing profiles of chemicals (Fig. S1). PCA could be used to describe the majority of observed variation (>73%) among samples by use of 2 components (Fig. S5). Classification of samples as 'toxic' (i.e. observed IC20) or 'non-toxic' (i.e. no observed IC20) revealed no significant structure in the data since there was no distinct clustering of the two groups (Fig. S6). Additionally, the data revealed an association of chemical classes O_4^+, SO_3^+, SO_4^+ and NO_3^+ with a greater overall toxicity rank (i.e. less toxic samples). Naphthenic acids were detected in all samples of OSPW, however the non-toxic extract F2-NE1 had relatively low abundances of NAs. Similarly, the F3-NE2b extract had low abundances of NAs but has been demonstrated to contain chemical classes which are bioaccumulative and toxic (Zhang et al., 2016; Morandi et al., 2015; 2016). This evidence supports a role for NAs in the toxicity of the dissolved organic fraction of OSPW as demonstrated previously (Scarlett et al., 2013; Morandi et al., 2015; Hughes et al., 2017). Chemical classes SO_3^-, SO_4^-, NO^+, O_2NS^+, SO^+, ONS^+ and NO_3^- were correlated with lesser toxicity, which is consistent with our previous results identifying the SO^+ and NO^+ chemical classes in the toxic extracts (Morandi et al., 2015, 2016). Samples F1-NE, F1-NE1 and F2-NE2 were clustered, which was consistent with previous results using biological responses (Fig. 1 and Fig. S3). Regardless, the inability of PCA to cluster chemicals in a similar way as analysis based on biological pathway is not surprising since it is known that chemicals behave different chemically when present as mixtures compared to when they exist independently (Bataineh et al., 2006; Cedergreen, 2014).

4. Conclusions

The dissolved organic fraction of OSPW is responsible for the majority of toxicity of OSPW, yet mechanistic studies investigating molecular mechanisms of OSPW have focused on a limited number of endpoints or results might have been confounded by potential interactions of the complex mixture e.g. high salinity. Therefore, it was instructive to screen extracts of OSPW re-suspended in laboratory control water by use of the LCA system to gain a greater understanding of molecular mechanisms of toxicity and to identify a profile of gene expression indicative of exposure to acutely toxic extracts of OSPW.

Genes indicative of general stress, protein damage and DNA damage were identified as uniquely responsive to acutely toxic extracts of OSPW. A general down-regulation of catabolic and anabolic processes was observed and are indicative of general non-specific toxicity. Changes to the expression of multiple genes and biological processes/KEGG pathways were indicative of changes to

the redox state of the cell, response to oxidative stress and are consistent with previous results across a range of extracts and species (He et al., 2012a; Wiseman et al., 2013a,b; Wiseman et al., 2013a,b; Zhang et al., 2011). Additionally, findings here support previous work that sulphur and nitrogen containing chemical classes are responsible for toxic potencies of extracts of OSPW (Alharbi et al., 2016; Morandi et al., 2016, 2015). Responsive genes, biological and KEGG pathways did not demonstrate a clear distinction among fractions, this was especially apparent for tertiary fractions (F3-NE2a and F3-NE2b), which had previously been hypothesized to have differing mechanisms of action.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.08.025.

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Elucidating mechanisms of toxic action of dissolved organic chemicals in oil sands process-affected water (OSPW)

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Characterization of Fractions by HPLC-Orbitrap-uHRMS.

Profiles of organic compounds in fractions were determined by use of LC-UHRMS according to methods described by Pereira et al (2013). Chromatographic separation was performed by use of an HPLC Transcend system (Thermo Fisher Scientific), consisting of a degasser, a 1250 bar quaternary pump, an auto-sampler, and a column oven. Separation was performed on a Cosmosil C18 MS-II column (100 x 3.0 mm, 2.5 µm particle size) (Nacalai USA, San Diego, CA, USA) at 40 °C. A flow rate of 0.5 mL/min and an injection volume of 3 µL were used in all analyses. Mobile phases consisted of (A) 0.1% acetic acid in water, and (B) 100% methanol. The mobile phase composition was 5% B for 1 min, followed by a linear gradient ramp to 90% B at 9 min, to 99% B over 5 min, and returning to 5% B in 1 min followed by a 4 min hold prior to the next injection. For figures and statistical analysis, total ion counts were summed for individual chemical classes.

Tables.

Table S1. Labels used to identify biological processes or KEGG pathways for PCA analysis.

PCA ID	GO ID	GO Term
BP1	GO: 0016051	carbohydrate biosynthetic process
BP2	GO: 0007154	cell communication
BP3	GO: 0008652	cellular amino acid biosynthetic process
BP4	GO: 0043094	cellular metabolic compound salvage
BP5	GO: 0006732	coenzyme metabolic process
BP6	GO: 0051188	cofactor biosynthetic process
BP7	GO: 1901657	glycosyl compound metabolic process
BP8	GO: 0018130	heterocycle biosynthetic process
BP9	GO: 0034220	ion transmembrane transport
BP10	GO: 0048523	negative regulation of cellular process
BP11	GO: 0034655	nucleobase-containing compound catabolic process
BP12	GO: 0009311	oligosaccharide metabolic process
BP13	GO: 1901615	organic hydroxy compound metabolic process
BP14	GO: 1901576	organic substance biosynthetic process
BP15	KEGG id: 00030	Pentose phosphate pathway
BP16	GO: 0071822	protein complex subunit organisation
BP17	GO: 0050794	regulation of cellular process
BP18	GO: 0009266	response to temperature stimuli
BP19	GO: 0009636	response to toxic substance
BP20	GO:0044283	small molecule biosynthetic process

Table S2. Genes differentially expressed in *E. coli* exposed to fractions of OSPW previously identified as responsive to environmental stressors (Gou et al., 2010). **1 column fitting image.**

Sample	Stress pathway	Gene
F1.Pool	*General function/ energy stress*	*bolA, crp*
	Protein stress	*clpB*
F1.NE	*Cell killing*	*rpoD, relB*
	Detoxification	*sodC*
	Protein stress	*clpB, dnaK*
	Redox stress	*soda, oxyR*
	SOS response/ DNA repair	*ssb*
F2.NE1	*Cell killing*	*rpoD, relB*
	Detoxification	*sodC*
	Protein stress	*clpB, dnaK*
	Redox stress	*soda, oxyR*
	SOS response/ DNA repair	*ssb*
F2.NE2	*Cell killing*	*rpoD, relB*
	Detoxification	*sodC*
	Protein stress	*clpB, dnaK*
	Redox stress	*soda, oxyR*
	SOS response/ DNA repair	*ssb*
F3.NE2a	*General function/ energy stress*	*bolA, crp*
	Protein stress	*clpB*
	Drug resistance	*sodC*
F3.NE2b	*General function/ energy stress*	*bolA, crp*
	Protein stress	*clpB*

Table S4. Labels used to identify chemical classes detected in extracts of OSPW for PCA analysis.

PCA ID	Chemical Class
CC1	O^-
CC2	O_2^-
CC3	O_3^-
CC4	O_4^-
CC5	SO^-
CC6	SO_2^-
CC7	SO_3^-
CC8	SO_4^-
CC9	NO^-
CC10	NO_2^-
CC11	NO_3^-
CC12	NO_4^-
CC13	ONS^-
CC14	O_2NS^-
CC15	O^+
CC16	O_2^+
CC17	O_3^+
CC18	O_4^+
CC19	SO^+
CC20	SO_2^+
CC21	SO_3^+
CC22	SO_4^+
CC23	NO^+
CC24	NO_2^+
CC25	NO_3^+
CC26	ONS
CC27	O_2NS

92 **Figures**

93

94

B)

A)

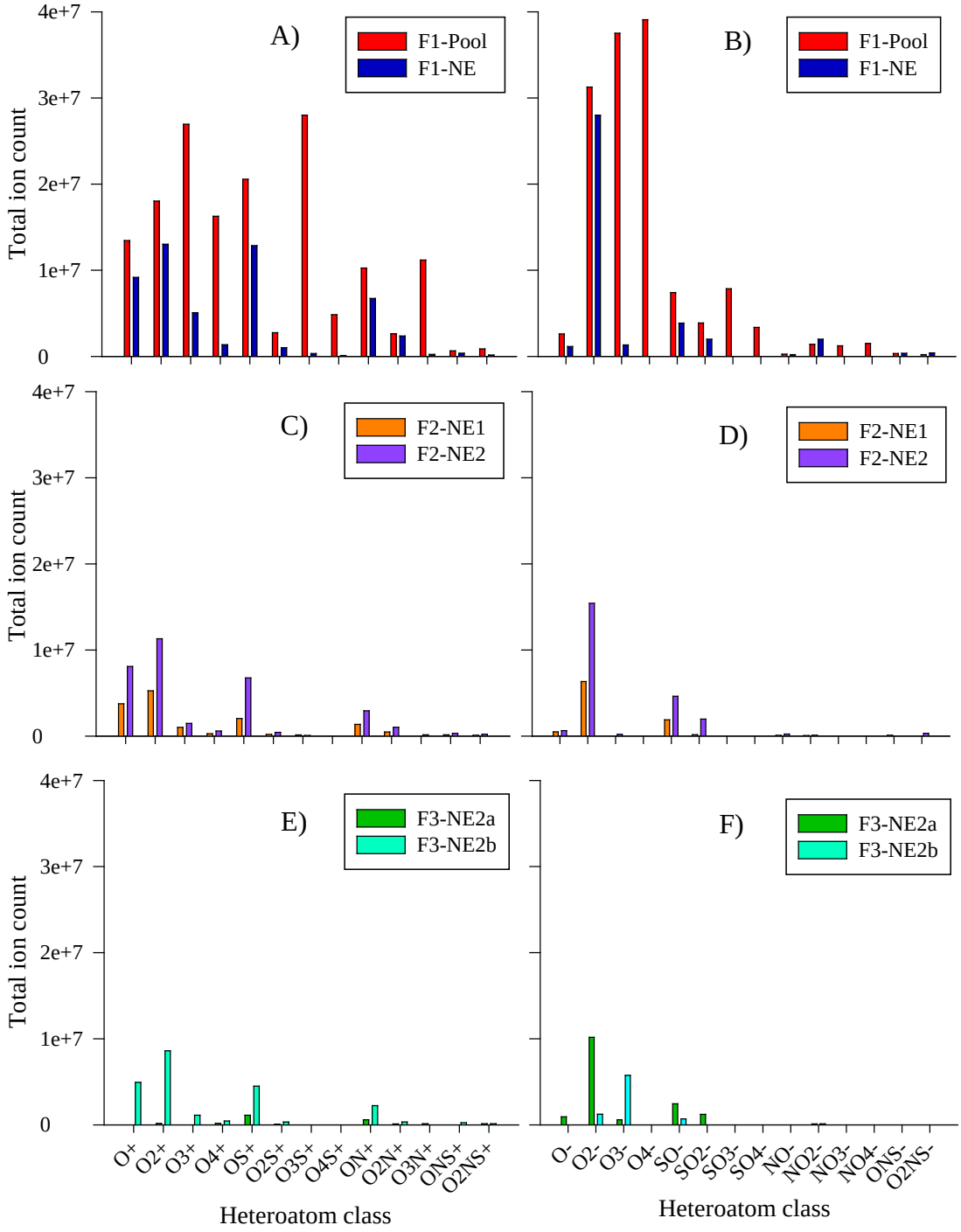
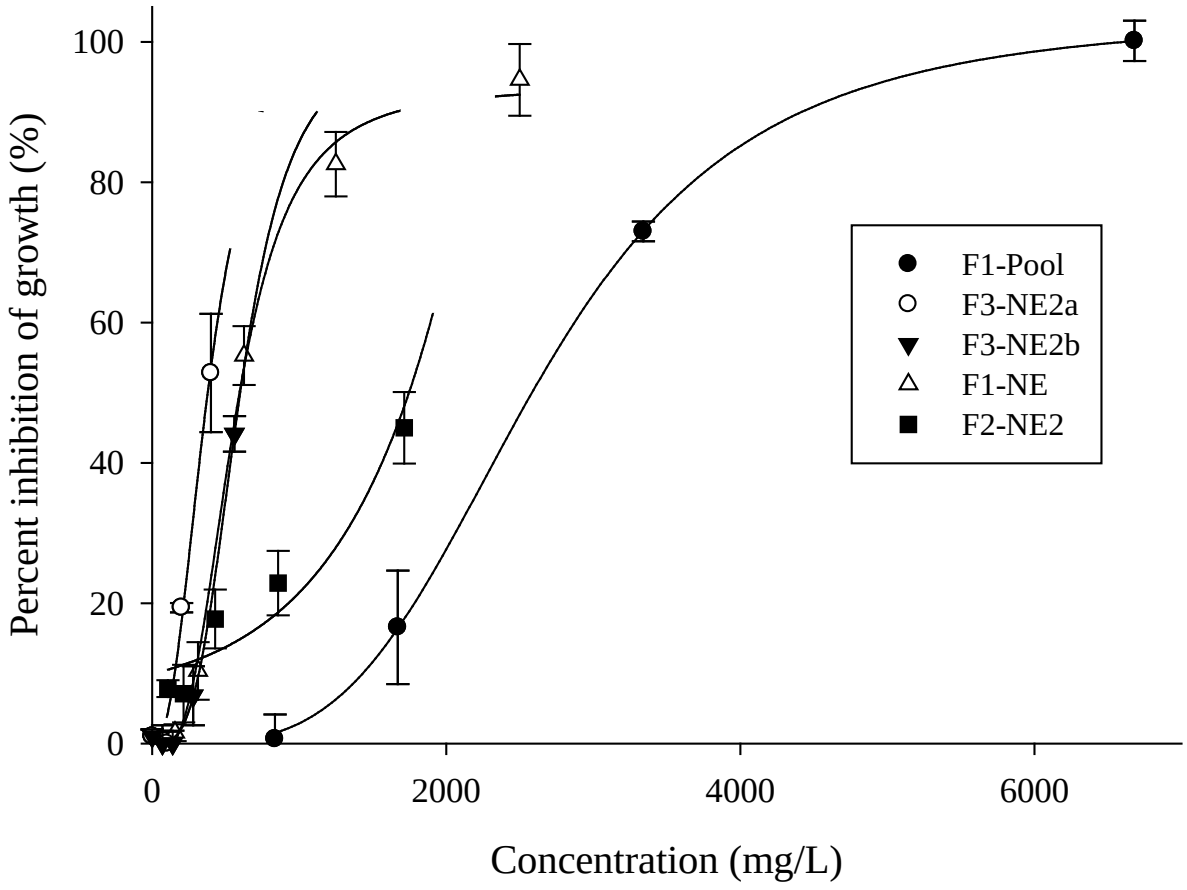


Figure S1. Total ion count for chemical classes detected in extracts of OSPW, A) Primary fractions in ESI+, B) Primary fractions in ESI-, C) Secondary fractions in ESI+, D) Secondary fractions in ESI-, E) Tertiary fractions in ESI+, F) Tertiary fractions in ESI-.

99 .



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102 Figure S2. Percent inhibition of growth of *E. coli* cells exposed to extracts of OSPW. **1-column**

103 **fitting image.**

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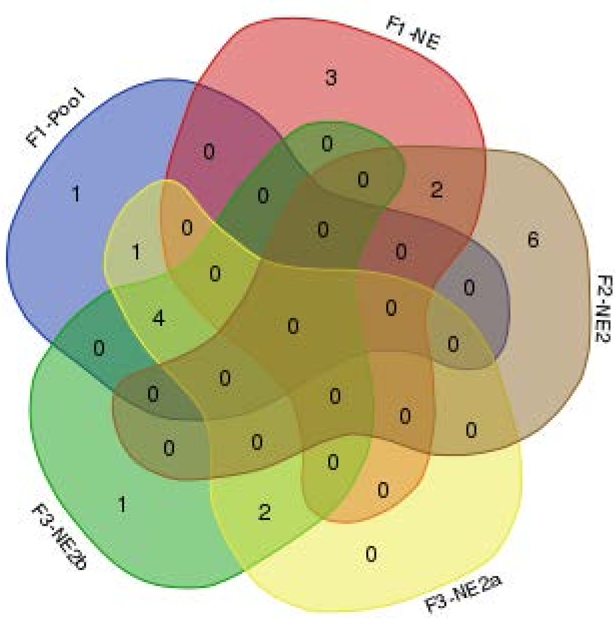

F1-Pool
F1-NE
F2-NE2
F3-NE2a
F3-NE2b

Figure S3. Venn diagram of biological processes or KEGG pathways identified as responsive in *E. coli* exposed to acutely toxic fractions of OSPW. **1 column fitting image.**

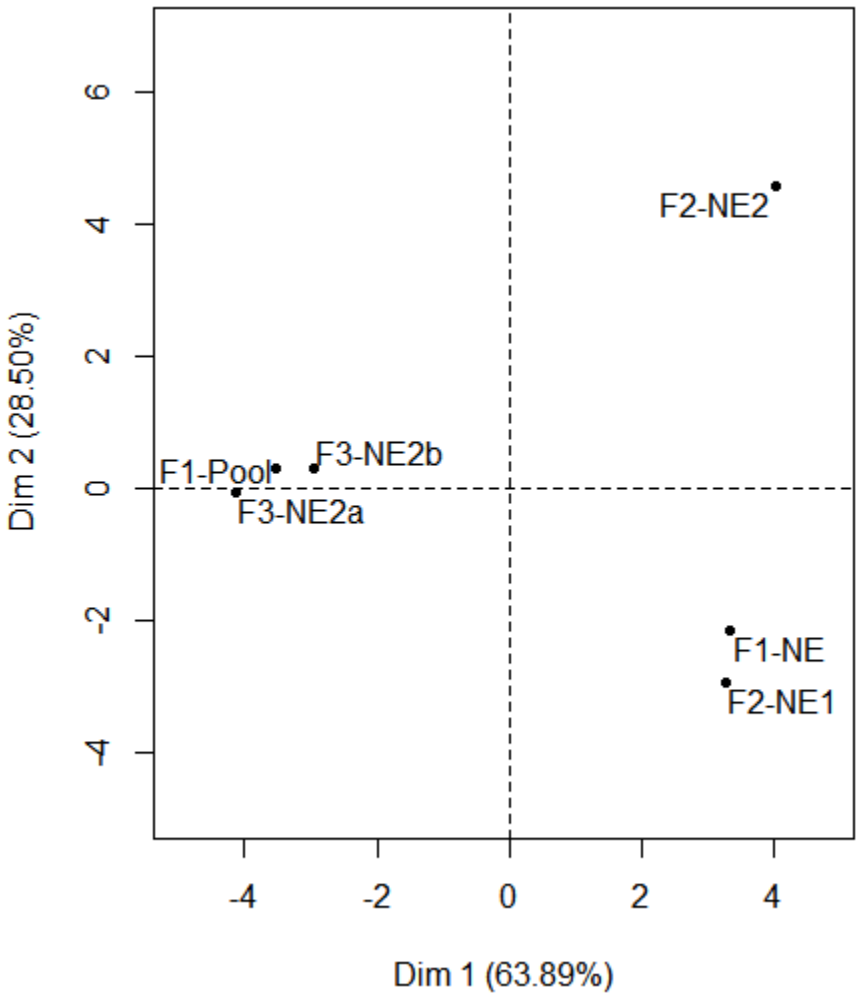
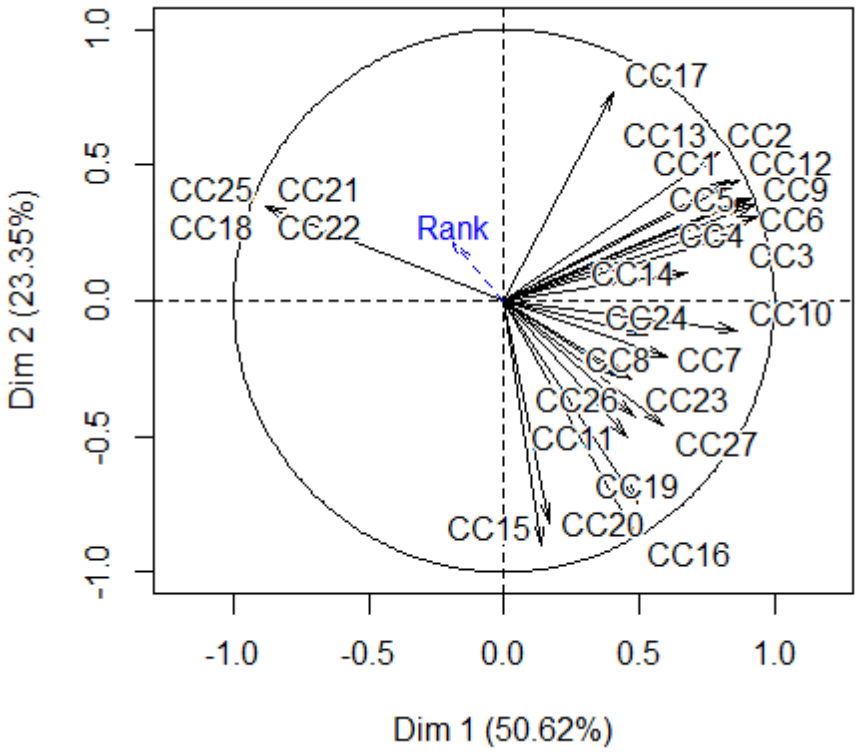


Figure S4. Individual factor map of extracts of OSPW, clustered by use of identified BP.

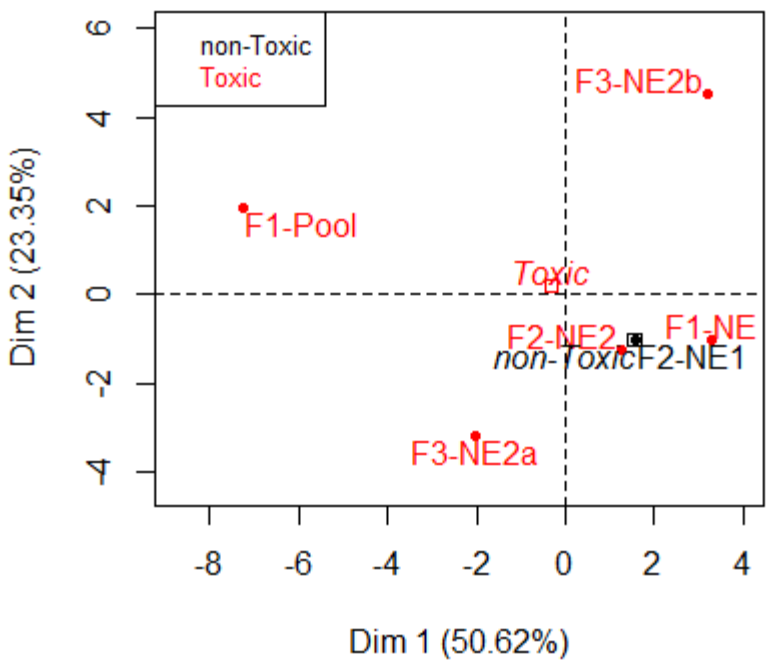


126

127 Figure S5. Variable factor map of chemical class ion counts for extracts of OSPW. Chemical

128 class labels are presented in Table S4. **1 column fitting image.**

129



130

131 Figure S6. Individual factor map of extracts of OSPW, clustered by use of identified chemical

132 classes.

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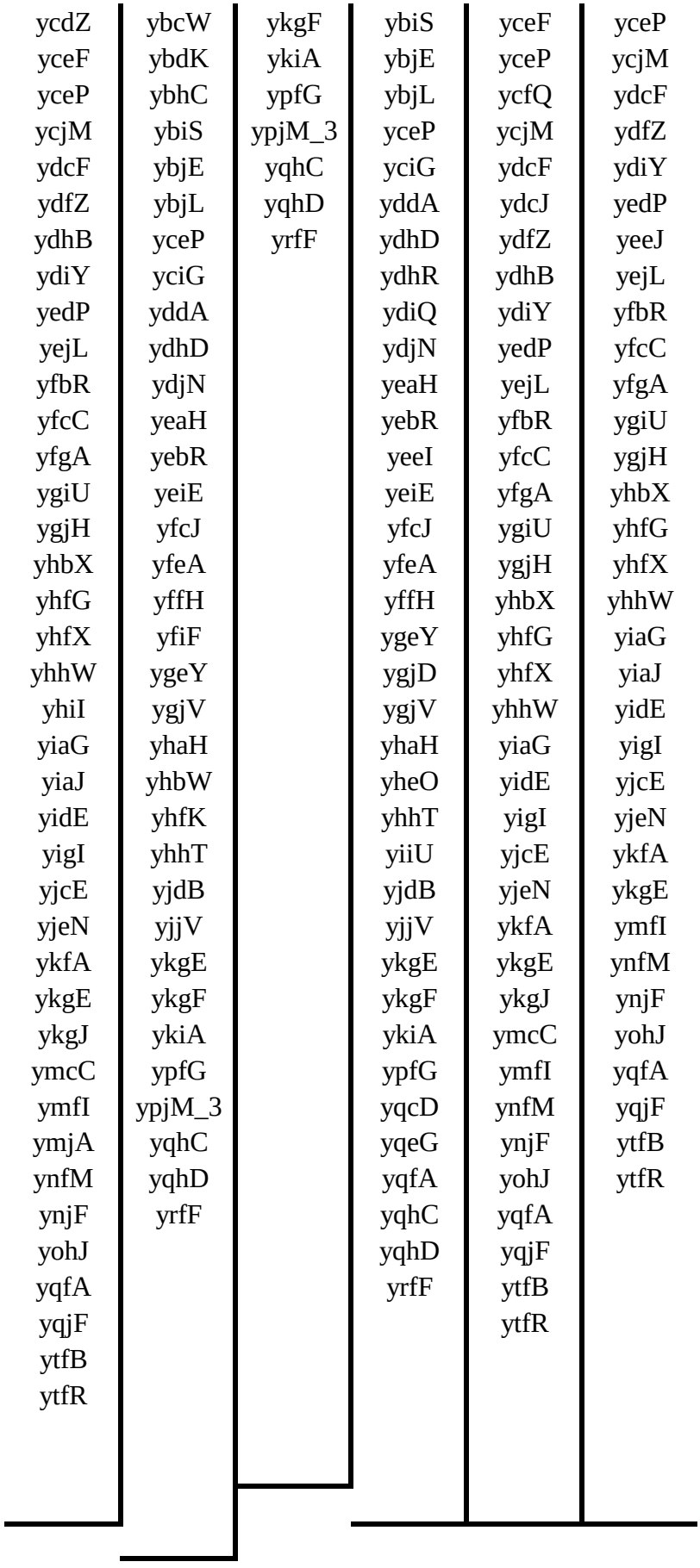
Table S1. 1.5-fold differentially expressed genes of *E.coli* exposed to extracts of OSPW.

F1-Pool	F1-NE	F2-NE1	F2-NE2	F3-NE2a	F3-NE2b
add	adhE	aer	adhE	add	add
adhE	adrA	allS	adrA	adhE	adhE
aldA	aer	ansB	aer	aldA	aldA
apt	allS	araD	allS	apt	apt
aspA	ansB	atpI	araD	aspA	aspA
b0663	araD	b3007	araE	b0663	b0663
bolA	argI	bax	argI	bolA	bolA
brnQ	asd	betT	atpI	brnQ	brnQ
cdd	atpI	clpX	b3007	cdd	cdd
clpB	b3007	corA	bax	clpB	clpB
clpS	betT	cysZ	betT	clpS	clpS
cpdB	chaB	dapA	chaB	cmr	cmr
crp	clpB	dapF	clpB	cpdB	cpdB
ddlA	clpX	def	clpX	crp	crp
deoB	corA	dhaR	corA	ddlA	ddlA
deoC	creB	dnaK	creB	deoB	deoB
dksA	cysZ	ecfK	cysZ	deoC	deoC
dsbG	dapA	eutR	dapA	dksA	dksA
emrR	dapF	fdrA	dapF	dppA	dppA
fkpB	def	flgB	def	dsbG	dsbG
frdA	dhaR	ftsZ	dhaR	emrR	emrR
gadW	ecfK	galS	dnaK	fkpB	fkpB
glpA	elaA	glmU	ecfK	frdA	frdA
glyQ	eutR	glnB	eutR	gadW	gadW
gntP	fdrA	glnU	fdrA	glpA	glpA
gpt	fecI	gntT	flgB	glyQ	glyQ
hdhA	flgB	hisL	ftsZ	gntP	gntP
hemH	ftsZ	ibpB	galS	gpt	gpt
insC-4	gadW	ileV	glmU	hcaT	hcaT
insE-4	galS	ilvI	glnB	hdhA	hdhA
ispB	gatR_1	ilvY	glnU	hemH	hemH
kdgR	glmU	insC-7	gntT	insC-4	insC-4
kil	glnU	iscR	hrpA	insE-4	insE-4
lacZ	gntT	kdtA	ibpB	ispB	ispB
lipA	hisL	lacI	ilvI	kdgR	kdgR
lpxP	hrpA	lacZ	ilvY	kil	kil
mcrA	ibpB	lgt	insC-7	lacZ	lacZ
menF	ileV	lolA	insE-3	lipA	lipA
menG	ilvI	map	kdtA	lpxP	lpxP
mokB	insE-3	mazG	lacI	mcrA	mcrA
murC	iscR	mcrB	lacZ	menF	menG

nanT	kdgR	mfd	lgt	menG	mgsA
nohB	kdsB	mog	lolA	mgsA	mokB
nupG	kdtA	otsB	mcrB	mokB	murC
pdxH	lacI	oxyR	mfd	murC	nohB
pgi	lacZ	pyrH	mog	nohB	nupG
pheP	lgt	queD	ompC	nupG	pdxH
phnC	lolA	racR	otsB	pdxH	pgi
pitA	map	rfaH	oxyR	pgi	pheP
pldA	mazG	rhsD	pldA	pheP	phnC
plsB	mcrB	ribC	pntA	phnC	pitA
pntA	mfd	rpoD	prfC	pitA	pldA
potA	mog	serA	pyrH	pldA	plsB
pspF	otsB	sfmA	racR	plsB	pntA
pykF	oxyR	torT	rfaH	pspF	potA
pyrB	phoA	ubiG	rhsD	pyrB	pspF
rcsF	pldA	ucpA	ribA	ribE	pykF
ribE	pntA	yacH	ribC	rimL	rcsF
rimL	pyrH	yacL	rmuC	rluB	rluB
rluB	queD	yaeH	rpoD	rrlE	rrlE
rrlE	racR	yafD	sdaA	rumA	rumA
rumA	relB	yagG	serA	serA	sieB
serA	rhsD	yagT	sodA	sieB	smpA
sieB	ribA	ybaP	sodC	smpA	speE
smpA	ribC	ybdK	ssb	speE	sscR
speE	rmuC	ybdL	sufI	sscR	talB
sscR	rpoD	ybhC	tesB	talB	tktA
talB	sdaA	ybjL	tolB	tktA	tufA
tktA	serA	yciG	torT	tufA	tyrP
tufA	sodA	ydhD	trxC	tyrP	ubiX
tyrP	sodC	ydjN	tyrP	ubiX	uspF
ubiX	ssb	yebR	ubiC	uspF	uspG
uspF	tolB	yeiE	ubiG	uspG	wrbA
uspG	torT	yfcJ	ucpA	wrbA	xseB
wrbA	tufA	yfeA	yacH	xseB	yabN
xseB	ubiC	yfeC	yacL	yabN	ybdL
yabN	ubiG	yffH	yaeH	ybdL	ybeB
ybdL	ucpA	yfiF	yafD	ybeB	ybgD
ybeB	yacH	ygeY	yagT	ybgD	ybjE
ybgD	yacL	yhaH	ybaP	ybjE	ybjN
ybjE	yaeH	yheO	ybcW	ybjN	yccA
ybjN	yafD	yhhT	ybdK	yccA	ycdC
yccA	yagG	yjdB	ybdL	ycdC	ycdZ
ycdC	ybaP	yjjV	ybhC	ycdZ	yceF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ykgE
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yfeA
yffH
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